CIRCULAR DICHROISM SPECTRA OF α-LACTALBUMIN

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SUMMARY

1. The circular dichroism spectra of α-lactalbumin have been obtained over a wide pH range. The near ultraviolet elipticity bands of the protein arise from transitions of asymmetric tryptophan and cystine residues. A contribution from tyrosine residues is not discernible because of overlap of its elipticity bands with tryptophan and cystine.

2. Based on the far ultraviolet elipticity bands, a tentative conformation of the polypeptide chain of α-lactalbumin has been proposed which contains 25–26% helix, 14–15% beta structure, and 60% unordered structure.

3. Significant changes occur in the near and far ultraviolet elipticity bands above pH 9 and below pH 4, and indicate an increase in α-helix content, and greater freedom of movement of side chains in the denatured protein compared to the native protein.

INTRODUCTION

Previous studies with bovine α-lactalbumin have demonstrated the occurrence of conformational changes at acid and alkaline pH (refs. 1, 2) and have shown that the processes leading to acid and alkaline denaturation are similar in many respects and result in apparent swelling of the protein and changes in the environment of tryptophan side chains[2–4]. A study of the optical rotatory dispersion (ORD) of α-lactalbumin[5] indicated that acid and alkaline denaturation does not involve a loss of α-helical structure of the protein but results primarily in changes in the environment of asymmetric aromatic chromophores and possibly cystine side chains. A limited study of the far ultraviolet circular dichroism (CD) spectra of native and acid denatured α-lactalbumin[6], also suggested that denaturation results primarily in a loss of tertiary structure.

To obtain a further knowledge of the structure of native α-lactalbumin and the

Abbreviations: CD, circular dichroism; ORD, optical rotatory dispersion.

conformational changes leading to its denaturation, we have measured its circular dichroism spectra over a wide range of pH. The work reported here confirms the results of previous investigations and provides additional information on the processes leading to denaturation as well as the nature of the asymmetric side chains in α-lactalbumin.

EXPERIMENTAL

α-Lactalbumin was prepared by the method of ROBBINS AND KRONMAN[7]. Protein concentrations were measured on a Cary Model 15 spectrophotometer and were so chosen to yield optical densities no greater than 1.0 at any wavelength. However, in those experiments in which concentration dependency was determined, the optical densities did not exceed 1.5. The solvent was 0.15 M KCl. An extinction coefficient of $E_{cm}^{1\%} = 20.1$ (280 mμ at pH 6.0) in 0.15 M KCl for α-lactalbumin was used[1]. Measurements of pH were made with a Radiometer 4 pH meter and solutions adjusted to the desired pH with 1 M KOH or 1 M HCl.

Circular dichroism measurements of α-lactalbumin were obtained with a Cary 60 recording spectropolarimeter fitted with a Model 6001 CD attachment. Measurements were made in cells of 1 mm and 10 mm at 25°. In the case of samples at acid pH (2–3.8) or alkaline pH (10–12.5) measurements were completed within at least 30 min after pH adjustment. This procedure gave reproducible results. The results are reported in terms of mean residue elipticity $[\theta]$, in units of degrees · cm²/decimole. A mean residue weight of 118 was used for purposes of calculation.

RESULTS

Near ultraviolet region

The near ultraviolet circular dichroism spectra of α-lactalbumin at various pH values are shown in Figs. 1 and 2. The spectra of the native protein in the pH range 5.4–8.0 are quite complex and independent of pH. The elipticity bands are negative, and the spectra are characterized by a small peak centered near 296 mμ, a trough near 252 mμ and a broad spectral envelope extending from about 255 mμ and a maximum near 272 mμ. The envelope also contains a number of plateaus and inflections. These spectra are consistent with the presence of optically active cystine and aromatic chromophores[8].

At pH 9 and higher the spectra are pH dependent and changes in elipticity with pH at the most prominent extrema along the spectral curve, 252, 272 and 296 mμ are shown in Fig. 3. A generalized reduction occurs in the spectral bands above 260 mμ when the pH is raised to 9 with 75–80% loss in elipticity occurring between pH 10.2 and 11.5 where denaturation of α-lactalbumin occurs[2]. The elipticity at the negative trough near 252 mμ also decreases as the pH is raised, but reverses its sign between pH 10.5 and 11 and becomes a positive peak (Fig. 2).

The nature of the spectral changes occurring at alkaline pH with α-lactalbumin are not consistent with the presence of asymmetric tyrosine side chains, since their ionization would result in a decrease in elipticity in the region of 275 mμ and a corresponding increase near 295 mμ (ref. 8). However, since these changes occur in the pH
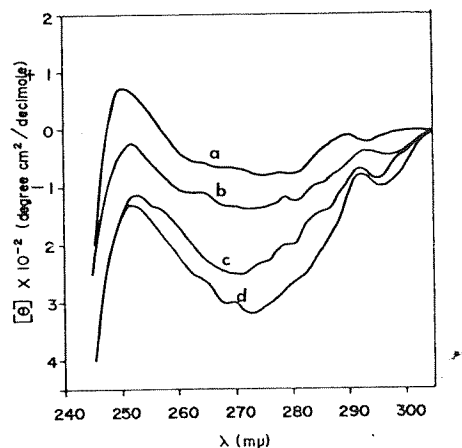
Fig. 1. Near ultraviolet circular dichroism spectra of α-lactalbumin. a, pH 11.5–12.5; b, pH 10.5; c, pH 9.4; d, pH 5.4–8.0.
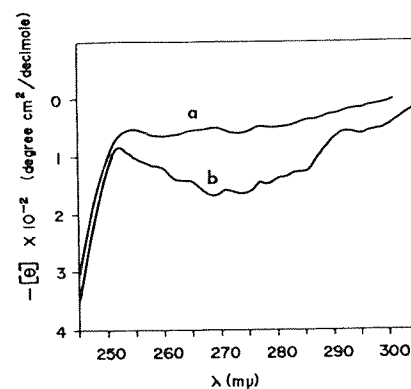
Fig. 2. Near ultraviolet circular dichroism spectra of α-lactalbumin. a, pH 2.0–3.5; b, pH 3.85.

range where denaturation takes place, they cannot reflect the conformation of the tyrosine side chains in the native protein.

At acid pH spectral measurements were limited to the pH range 3.85–2.0 due to the limited solubility of α-lactalbumin[1]. As seen in Figs. 1, 2 and 3 a generalized reduction in elipticity occurs similar to that observed at alkaline pH. The spectral changes are complete by pH 3.5 and the elipticity bands of the acid denatured protein are reduced over 80% as compared to the native protein. As at alkaline pH, the elipticity band located near 252 mμ is lowered, but does not change sign (Figs. 1, 3).

Far ultraviolet region

The far ultraviolet circular dichroism spectra at various pH values are shown in Figs. 4 and 5. The spectra at pH 7 and at acid pH (2–3) are similar to those previ-
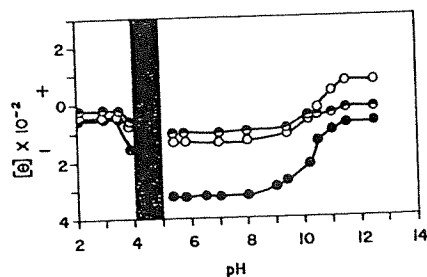
Fig. 3. The pH dependency of the ultraviolet elipticity of α-lactalbumin at various wavelengths. ○, 252 mμ; ●, 272 mμ; ◐, 295 mμ.
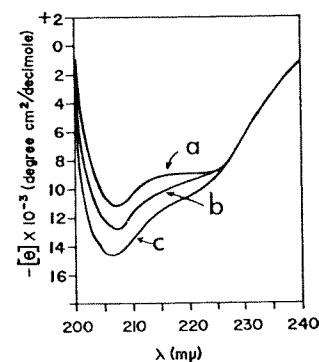
Fig. 4. Far ultraviolet circular dichroism spectra of α-lactalbumin. a, pH 5.4–5.8; b, pH 3.5–3.85; c, pH 2.0–3.0.
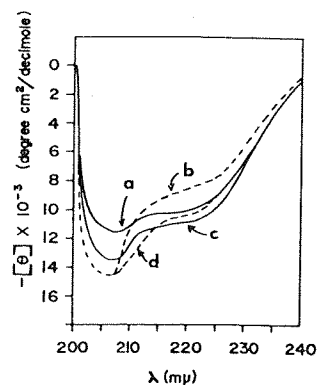
Fig. 5. Far ultraviolet circular dichroism spectra of α-lactalbumin. a, pH 7.0; b, pH 12.5; c, pH 10.0; d, pH 11.3.
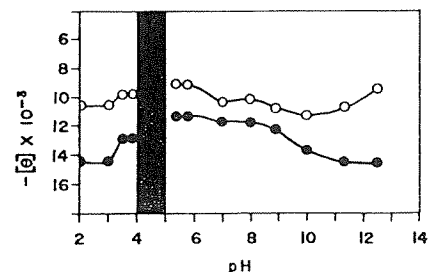
Fig. 6. The pH dependency of the far ultraviolet elipticity at various wavelengths. $\bigcirc$, 220 mμ; $\bullet$, 208 mμ.

ously reported by KRONMAN[6] for α-lactalbumin at these pH values. The spectra of the native protein in the pH range 5.4 to 9 are characterized by a negative peak located near 208 mμ and a relatively flat region of negative elipticity extending from about 212–223 mμ. The general shape of the spectral envelope in the region from 200 to 240 mμ changes little in this pH range; however, the absolute magnitude of the elipticity of the negative extrema are somewhat pH dependent. This is shown in Fig. 6, where the elipticity at the 208-mμ peak and in the plateau region at 220 mμ are plotted as a function of pH. This behavior was not related to pH dependent association of α-lactalbumin[1,2], since spectra at concentrations ranging from 0.013 to 0.076% protein within this pH range show no significant differences. The results suggest that the pH dependency of the elipticity in the region pH 5–9 may be related to differences in interaction with solvent or charge effects rather than changes in protein conformation.

In the pH region below 4 and above 9 where denaturation occurs[1,2], the shape of the far ultraviolet spectra changes significantly. As shown in Figs. 4 and 5 the peak at 208 mμ becomes more prominent and increases in elipticity (Fig. 6) while the curve in the region of 212–230 mμ becomes steeper, suggesting significant changes in protein conformation. As shown in Fig. 6, the far ultraviolet elipticity changes occurring at acid pH are complete at pH 3, while at alkaline pH, changes still occur at pH 12.5.

We have evaluated the conformation of the polypeptide chain of α-lactalbumin at various pH values from the elipticity data in the region of 208–240 mμ, using the method (Method I) of GREENFIELD AND FASMAN[9]. This method was chosen since it was found to give reasonable results when applied to several proteins whose structures were known from X-ray crystallographic analysis, and which included hen lysozyme, a protein thought to be structurally similar to α-lactalbumin[10]. By this procedure an estimate of the α-helical content was obtained from the elipticity at 208 mμ (see Table I), and the percentage of antiparallel β structure (pleated sheet) and unordered structure was approximated from the estimated α-helical content by a visual com-

parison of the experimental CD curves with calculated CD curves obtained from GREENFIELD AND FASMAN[9]. The latter curves are constructed from a linear combination of varying percentages of the standard reference spectra of each conformation obtained from poly-L-lysine. The computations (Table I) show that the conformation of the polypeptide chain of native α-lactalbumin is quite constant in the pH range 5.4–9. In the pH region above 9 and below 4, where denaturation occurs, the conformational changes which take place result in an apparent increase in α-helix content from 25–26% to 36% and a decrease in β structure from 14–15% to 4% with the percentage of unordered structure remaining unchanged. From ORD measurements[5], the α-helical content of native α-lactalbumin was found to be about 21–23% when determined from the 233-mμ Cotton effect trough. At alkaline pH the α-helix content increased to about 27%; however, no change in helix content was noted at pH2.00. The CD and ORD measurements are therefore in qualitative agreement, except for the unexplained difference at acid pH. The similarity in secondary structure of acid and alkaline denatured α-lactalbumin suggested by these results as well as the pH dependency of the elipticity changes are also in agreement with previous studies[1-4]. By comparison the near ultraviolet CD spectra of these forms (Figs. 1 and 2) indicate that their side chain conformations differ.

DISCUSSION

The presence of asymmetric tryptophan in native α-lactalbumin can be inferred from the peak centered near 296 mμ and the general shape of the spectral envelope above 260 mμ (ref. 8). The pH-dependent lowering of the elipticity bands at 296 and 272 mμ (Fig. 3) indicates that the tryptophan residues which contribute to these bands have an increased freedom of movement in the acid and alkaline denatured protein, as compared to the native protein. This is in accord with pH dependent changes in other properties of α-lactalbumin such as fluorescence[2,4] and absorption difference spectra[3,4] which reflect the environment of tryptophan.

The spectral changes occurring at alkaline pH (Fig. 1) indicate the absence of asymmetric tyrosine in denatured α-lactalbumin. However, the presence of optically active tyrosine is not discernible in the native protein because of overlap of the elipticity bands of these side chains with those of tryptophan and cystine at neutral pH. Experiments now in progress in our laboratory involving the chemical modification of tyrosines in α-lactalbumin are expected to provide information on their optical activity in the native protein.

The elipticity changes occurring at 252 mμ (Fig. 3) could arise from cystine or tyrosine residues since both exhibit elipticity bands in this wavelength region[8]. These changes occur in the pH region of denaturation, however, and are therefore most reasonably ascribed to cystine, since asymmetric tyrosines are absent in the denatured protein. The elipticity band generated by disulfides in the near ultraviolet region is thought not to arise from the inherent asymmetry of the disulphide bridge, but from the asymmetry resulting from its interaction with vicinal atoms and solvent, with the sign of the band being a reflection of the environment in its vicinity[11]. This suggests that the observed pH dependency of this band in α-lactalbumin arises from conformational changes occurring in the vicinity of the disulphide bridges as a result of denaturation. The position of the band near 252 mμ suggests that the dihedral

angle of the sulphur atoms in the disulphide bridges is close to 90° (ref. 8), in agreement with the geometry of these groups in α-lactalbumin as deduced from model building[10].

The pH dependency of the far ultraviolet CD data (Fig. 6) confirm the results of hydrodynamic[1,2], titration[12] and ORD measurements[5] which indicate that the polypeptide backbone conformation of α-lactalbumin changes little between pH 4 and pH 9–10, but that outside this range structural changes occur resulting in denaturation. The similarity in the far ultraviolet CD spectra of acid (pH 2–3) denatured and alkaline (pH 11.3) denatured α-lactalbumin (Figs. 4c and 5d) indicate that the secondary structure of these two forms are similar and is in contrast to their side chain conformations which are somewhat different (Figs. 1 and 2). The difference may account for the observed difference in association and aggregation of these forms[1,2].

Our conclusions regarding the origins of the elipticity changes in the far ultraviolet spectra differ with those of KRONMAN[6] who suggested that they arise from loss of asymmetry of side chain chromophores. While a contribution from side chain chromophores to the far ultraviolet elipticity changes cannot be ruled out, it clearly cannot account for all of the observed changes. Thus the changes in the optical activity of the side chain chromophores of α-lactalbumin are complete by pH 3.5, as judged by the near ultraviolet CD bands (Fig. 3). By contrast only about 50% of the far ultraviolet elipticity changes occur by pH 3.5 (Fig. 6) with the remainder occurring below this pH and therefore reflecting only peptide transitions, presumably free of contributions from side chain chromophores. The supposition that side chain chromophores contribute little to the elipticity in the region of 208–240 mμ is also supported by PEGGION et al.[13] who have demonstrated that the CD spectra of synthetic polypeptides in this region are unaffected by the incorporation of up to 16% tryptophan residues.

TABLE I

ESTIMATED PERCENTAGES OF α HELIX, β STRUCTURE, AND UNORDERED STRUCTURE IN α-LACTALBUMIN

pH	α Helix* (%)	β Structure (%)	Unordered structure (%)
2–3	36	4	60
5.4–5.8	25	15	60
7–9	26	14	60
11.3	36	4	60

$$* = \frac{[\theta]_{208 \text{ m}\mu} - 4000}{33\,000 - 4000} \times 100.$$

The conformation of the polypeptide chain of native α-lactalbumin estimated from the far ultraviolet CD data (Table I) is similar to that found for lysozyme[9] by the same method (29% helix, 11 β structure, and 60% unordered structure) and is in agreement with the work of KRONMAN[6] who previously reported a similarity in the far ultraviolet CD spectra of the two proteins. In contrast to circular dichroism, which is essentially a measure of short range interactions, a recent study of the struc-

ture of α-lactalbumin and lysozyme by low angle X-ray diffraction[14], which measures both size and shape of molecules in solution, suggests that their structures are quite different.

Because of uncertainties in the dichroic properties of model conformations which can exist in proteins, and the empirical nature of the methods used for estimating their contribution, the polypeptide backbone conformation proposed for the native and denatured forms of α-lactalbumin should be considered tentative. The confirmation of its structure, and postulated similarity to hen lysozyme, must await the results of X-ray crystallographic analysis now in progress (quoted in ref. 10).

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